Laminin 2 controls mouse and human stem cell behaviour during midbrain dopaminergic neuron development

Laminin α2 controls mouse and human stem cell behaviour during midbrain dopaminergic neuron development

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Abstract

The development of the central nervous system requires the coordination of proliferation and differentiation of neural stem cells. Here, we show that laminin alpha 2 (lm-α2) is a component of the midbrain dopaminergic (mDA) progenitor niche in the ventral midbrain (VM) and identify a concentration-dependent role for lm211 in regulating mDA progenitor proliferation and survival via distinct set of receptors. At high-concentrations, lm211 rich environments maintain mDA progenitors in a proliferative state via integrins α6β1 and α7β1. Whereas low concentrations of lm211 support mDA lineage survival via dystroglycan receptors. We confirmed our findings in vivo where, in the absence of lm-α2, the VM was smaller, with increased apoptosis, and the progenitor pool depleted through premature differentiation resulting in fewer mDA neurons. In examining mDA neuron subtype composition we found a reduction in later-born mDA neurons of the ventral tegmental area, which control a range of cognitive behaviours. Our results identify a novel role for lm in neural development and provide a possible mechanism for autism-like behaviours and brainstem hypoplasia seen in some patients with mutations of the human lm-α2 gene.

Keywords: Extracellular matrix, neural stem cells, laminin, integrin, dystroglycan, dopaminergic neurons, congenital muscular dystrophy

Introduction

During development, the embryonic central nervous system (CNS) consists of proliferating neural stem cells (NSCs) that are exposed to a balance of intrinsic and extrinsic factors that regulate cell fate decisions (1,2). One such extrinsic determinant is the extracellular matrix (ECM), a multifunctional network of proteins interacting with and regulating a range of cell functions. Transcriptional analysis of mouse and human neocortices display an enrichment of ECM genes such as laminins (lms), proteoglycans and integrins in the germinal zones suggesting they may play a role in regulating NSC behaviour (3,4).

Lms are high-molecular weight, heterotrimeric glycoproteins composed of an α, β and γ chain. Currently 5α, 3β and 3γ chains have been identified combining to form at least 16 different combinations in the mouse, creating considerable tissue heterogeneity. Mutations to the human gene (lama2) encoding the lm-α2 protein results in merosin-deficient muscular dystrophy (MDC1A)(5–7). Whilst this congenital muscular dystrophy (CMD) results primarily in skeletal muscle damage, patients often exhibit a broad spectrum of neuroanatomical defects including white matter abnormalities, cerebellar cysts and brainstem (midbrain, pons and medulla) hypoplasia (8,9). However, the specific functions of lm-α2, particularly in the developing CNS, that lead to these brain abnormalities in MDC1A are unknown.

A further set of clinical symptoms seen in these patients includes neurological deficits in executive functions, intellectual disability and attention deficit hyperactive disorder (ADHD) (8–11). This phenotype highlights a possible involvement of the ventral domain of the midbrain, consisting of dopaminergic (mDA) neurons that can be subdivided into two main nuclei: substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). SNc mDA neurons are generated first, project largely to the striatum and contribute to the control of voluntary movement, with the selective death of these neurons being the pathological hallmark of Parkinson's disease (PD) (12). In contrast, later-born VTA mDA neurons innervate the hippocampus and the prefrontal cortex (PFC) regulating a range of cognitive functions (13). A dopamine imbalance in the VTA mDA neurons has been implicated in the aetiology of ADHD, obsessive-compulsive disorder (OCD), addiction and schizophrenia (14–16).

In this study, we have explored the role of lm-α2 protein in NSC development, focussing on the hypothesis that mutations in lama2 gene disrupt mDA neurogenesis resulting in a dopamine imbalance that may contribute to some of the neuropsychiatric deficits found amongst CMD patients. We first confirm the expression of lm-α2 in the human embryonic ventral midbrain (VM) during mDA neurogenesis. We then utilised a human embryonic stem (hES) cell model of mDA differentiation to define a functional role for lm-α2 in mDA progenitor proliferation and survival. Finally, we confirmed our findings in vivo using a lm-α2 null transgenic mouse model. Our findings show that lm-α2 regulates NSC behaviour by controlling the survival of mDA progenitors and their timely differentiation into neurons. In the absence of lm-α2, mDA progenitors are prematurely depleted resulting in a reduction in the number of late-born mDA neurons in the VTA.

Results

Lm-α2 is present in the developing human VM surrounding mDA progenitors

Using immunohistochemistry (IHC), chain-specific lm expression in the mesencephalon of human embryos at 6-10 post-conception weeks (pcw) revealed the early embryo (6 pcw) to be rich in lm-α2 (fig 1). The VM was immuno-positive for lm-α2 throughout the entire apico-basal axis surrounding a population of rapidly dividing (Ki67+) mDA progenitors. Analysis of a previously published single-cell RNA sequencing (scRNA-seq) study of the human VM between 6-11 pcw suggests that pericytes are a potential contributing cell source and could potentially secrete this lm into the interstitial space (fig 1) (17). A developmental time-course analysis revealed that protein expression diminished over time and by 10 pcw, lm-α2 expression was largely absent from the human VM. Similarly, in the mouse VM, lm-α2 protein expression rapidly diminished after E10.5 with little or no detectable lm-α2 at E14.5 (fig S1). The loss of interstitial lm-α2 expression correlated with the loss of proliferating mDA progenitors, suggesting a potential role for lm-α2 in supporting self-renewal. Examination of the spatiotemporal expression patterns of the remaining four lm-α chains revealed the basal laminae of the blood vessels within the VM to be rich in lm-α4 (fig S1), consistent with the scRNA-seq data showing expression to be greatest in endothelial cells and pericytes. Expression of the lm-α1 was most pronounced in the basement membrane lining the basal surface of the neural tube whereas lm-α5 could be seen on the ventricular surface and interspersed around the cells of the VM. This again is consistent with the scRNA-seq data showing expression of these laminin genes in radial glial cells (Rgl – whose processes span the two surfaces of the developing mesencephalon) and progenitors (fig S1). Lm-α3 protein and mRNA expression were absent in the midbrain floor plate, underlining the specificity of the different laminin expression patterns.

Lm211 regulates the balance between proliferation and differentiation in a concentration-dependent manner and controls the survival of human mDA progenitors

To assess the functional role of lm-α2, a hES cell model of mDA differentiation was established (fig S2) using a previously published protocol capable of yielding mDA neurons positive for a range of markers such as TH, Nurr1, DDC, Pbx1a, Girk2 and Calb1 (fig S2) (18). Day 14 VM progenitors that are positive for FoxA2+, Lmx1a+ and Otx2+ (fig S2), analogous to mDA progenitors in vivo at 6pcw, were incubated with the recombinant version of the lm-α2 containing trimeric protein, lm211, at a concentration ranging from 0.5-12 $\mu g ml^{-1}$ for a period of 14 days. The thymidine analogue, EdU, was administered prior to fixation to label proliferating cells and the cultures were stained for tyrosine hydroxylase (TH), which marks mDA neurons. Increasing concentrations of lm211 increased the number of mDA progenitors that were EdU+, with a concomitant reduction in the number of TH+ mDA neurons (fig 2A-C).

Focussing on two concentrations favouring proliferation (4 $\mu g ml^{-1}$) and differentiation (1 $\mu g ml^{-1}$), the differentiation dynamics were interrogated further. Cultures were fixed at day 21, 28 and 35 and were examined for proliferation (EdU), differentiation (Nurr1 and TH) and cell death (active caspase 3, aC3). No differences were detected at day 21 between either conditions; however, the lm211-rich environment (4$\mu g ml^{-1}$) sustained cells in a proliferative state (fig 2D) and consequently delayed differentiation resulting in fewer Nurr1+ post-mitotic neuroblasts (fig 2E) and TH+ neurons (fig 2F) being detected at day 28 and 35 compared to lm211-poor condition (1$\mu g ml^{-1}$). Whilst the number of apoptotic cells increased over the duration of the culture, no significant difference was detected between the two lm211 concentrations (fig 2G).

Having shown a role for lm211 in promoting VM progenitor proliferation, we next determined whether this property was specific to the lm-α2 isoform by treating the cultures with three other lm isoforms – lm111, lm411 and lm511 – at the previously identified concentrations (1 and 4 $\mu g ml^{-1}$). Rates of proliferation of mDA progenitors were the same on all lm isoforms, with no significant difference detected in EdU labelling at either concentration (fig S3A, B, D). Similarly, no effect on the number of TH+ cells was detected at high concentrations of lm-α isoforms (fig S3E). Surprisingly, however, a significant increase in the number of TH+ neurons was detected on lm211 at low concentration, compared to the other three isoforms (fig S3C). We therefore next determined whether this increase in TH+ neurons on low concentrations of lm211 was due to enhanced differentiation or survival by examining aC3 immunoreactivity (a marker of apoptosis) on the four different lm isoforms. Significantly fewer aC3+ cells were seen on lm211 (fig S3F) suggesting the increase in the number of mDA neurons is due to improved cell survival. Taken together, these results suggest lm211 has distinct concentration-dependent effects, with a lm-rich environment supporting proliferation and expansion of the progenitor pool irrespective of isoform, whilst low concentrations of lm-α2 containing isoform promote survival of mDA lineage cells.

Interaction of Lm211 and human mDA progenitors is mediated by distinct receptor engagement

To identify the cell surface receptors responsible for the different effects of lm-α2 on mDA progenitors, we used a functional blocking antibody approach. As the lm-rich environment maintains cells in a proliferative state irrespective of isoform, we speculated that the integrin family of receptors were involved. Three principle lm-binding integrins have been identified: α3β1, α6β1 and α7β1. All were expressed in the hVM; scRNA-seq analysis showed that the radial glia (hRgl) and midline progenitors (hProgM) express significant levels of α6 integrin and detectable levels of α7 and β1 integrins (fig S4). We further confirmed the presence of these three integrin subunits via immunostaining hES cell derived mDA progenitors (fig S4). Blocking the β1 integrin subunit resulted in a complete loss of adhesion and cell detachment (data not shown) whilst blocking the individual α-subunits had a negligible effect on mDA progenitor proliferation (fig 3B). As the integrin α-subunits may functionally compensate for each other, we next blocked pairwise combinations of α-subunits. Blocking α3 integrin had no discernible effect, but the combined blockade of both integrin α6 and α7, in the presence of $4 \mu g ml^{-1}$ lm211, resulted in the loss of the lm211-driven increase in EdU+ mDA progenitors and an increase in the number of TH+ mDA neurons (fig 3B and 3C). We further confirmed the role of integrin α6 and α7 in mediating the proliferative response by blocking both receptors in the presence of lm111, lm411 and lm511 at $4 \mu g ml^{-1}$ and successfully reversing the increase in proliferating mDA progenitors (fig S5).

As the previously seen increased cell survival of hES derived mDA lineage cells (fig S3C and G) was specific to low concentrations of lm-α2, we speculated that dystroglycan – a high-affinity lm-α2 receptor enriched in the floor plate, point of origin of mDA neurons, may be mediating the effect (19–21). We confirmed the expression of dystroglycan in the hVM via immunostaining and also in the scRNA-seq dataset, in which it is expressed at significant levels in hRgl (fig S4). Blocking dystroglycan with a targeted antibody (IIH6C4) in vitro reversed the laminin-isoform specific increase in TH+ neurons seen on low-concentrations of lm211 to levels comparable to those seen on lm111 (fig 3D-F). When we administered the antibody without any exogenous lm211, we did not observe any significant differences in TH+ cells confirming the lm-specific nature of the interaction (fig S5).

A loss of progenitors and premature differentiation results in reduced number of neurons in the ventral midbrain of Lm-α2 null mice

Together, results using human cells and tissue suggest the hypothesis that the fall in lm-α2 expression levels in the human VM between 6 and 10pcw controls VM NSC cell cycle exit and mDA neuron differentiation, with the lower concentrations of lm-α2 then supporting cell survival. To confirm that lm-a2 is such an instructive component of the niche in vivo, we examined the VM of lm-α2 null mouse embryos during mDA neurogenesis at embryonic (E) day 10.5-14.5, corresponding to 6-10pcw in humans. We first confirmed that the lm-α2 null embryos do not express lm-α2 (fig S6).

Our hypothesis predicts that in the absence of lm-α2 there would be fewer mDA progenitors and more neurons due to the loss of the proliferative effect of this laminin, resulting in premature differentiation. This was confirmed; the *lama2*$^{-/-}$ embryos were smaller than the WT littermate controls and the ventral midbrain (VM) domain, demarcated by FoxA2 expression, consisted of significantly fewer cells (fig S7A-D). Premature differentiation at E10.5 could be detected in the lama2$^{-/-}$ embryos as evidenced by increased number of Nurr1+ and TH+ cells at this age, with a concomitant reduction in proliferating VM progenitors (double Ki67+ and FoxA2+ cells) (fig 4A-D). Nascent mDA neurons were seen not only in the marginal zone along the basal surface of the VM but also in ectopic positions at the ventricular surface of the lama2$^{-/-}$ VM (fig S8) – a region where the high concentration of lm-α2 would normally prevent differentiation.

To exclude the possibility that the observed reduction in size of the VM resulted from patterning defects rather than changes in proliferation, we examined the position of cells expressing FoxA2, Lmx1a or Corin and calculated the area of their domains, normalised to the area of the ventricle. No significant difference in position or domain sizes was seen at E10.5 or E12.5 (fig S7), suggesting patterning is unaffected in the mutant embryos. We also examined the expression patterns of Nkx6-1 and *Wnt1* in wild-type and mutant embryos at E12.5 and found no differences (fig S7H,I). We did however note a slight delay in the lateral expansion and medial inhibition of Shh expression, with low levels of Shh in the mutant basal plate compared with the controls (fig S7I). This finding is in agreement with both the known function of Shh to control the expansion of the VM (22,23) and with our findings of decreased number of FoxA2^{+} cells (figS7K), impaired growth and reduced proliferation in the VM.

Our original hypothesis also predicts increased levels of apoptosis due to the loss of the survival-promoting effect of low concentrations of lm-α2. Again, this was confirmed; by E12.5 numerous active caspase 3+ (aC3) cells were found along the border of the ventricular zone and intermediate zone that separates progenitors from postmitotic neuroblasts, suggesting that apoptosis takes place during cell-cycle exit (fig 4E). Quantification revealed a near two-fold increase in aC3+ cells and fewer FoxA2+ progenitors (double Foxa2+ and KI67+ cells) in the VM (fig 4F, J).

Ectopic TH+ neurons were once again detected at the ventricular surface of the mutant VM at E12.5 (fig S8). However, in contrast to the situation at E10.5, by E12.5 the intermediate and marginal zone of the *lama2*$^{-/-}$ embryos contained fewer Nurr1+ and TH+ cells (fig 4H

and I). To determine the cause of this reduction in neuron number, we next examined the expression of the proneural gene Ngn2 in Lmx1a+ mDA progenitors (24). Whilst these experiments confirmed the reduction of midbrain floor plate mDA progenitors, identified as Lmx1a+, we found no significant difference in the subset of progenitors undergoing neurogenesis, identified as Ngn2+, in the lama2$^{-/-}$ embryos (fig 4L). Consequently, we conclude that the proportion of progenitors undergoing neurogenesis is greater in the mutant embryos, leading to accelerated differentiation and depletion of the progenitor pool. To test this directly, we injected pregnant mice subcutaneously with EdU at E11.5 and dissected the embryos 24 hours later at E12.5. We then quantified the number of dividing cells (EdU+) that had exited cell cycle and were positive for the post-mitotic marker Nurr1. Confirming our previous results, we found a significant increase in neurogenesis with a 26% increase in the number of cells positive for both EdU and Nurr1 in the mutant embryos compared with wild-type littermate controls. Meanwhile, there was a significant decrease in the number of Lmx1a+ EdU+ cells that are Nurr1- (i.e. mDA progenitors that are still in cell cycle). Consistent with this, by E14.5, there were significantly fewer TH+ neurons in the mutant embryos than in the WT littermate controls (fig S9).

To determine whether these effects are restricted to the mDA lineage, we next examined non-mDA neurons in the VM. Brn3a is a marker of neurons within the red nucleus, immediately lateral to the mDA neuron domain. The number of Brn3a+ neurons in *lama2*$^{-/-}$ mutants were significantly reduced compared to wild-type littermate controls at E14.5 (fig S10), indicating that basal plate neurogenesis is also impaired.

Lm-α2 null mice have fewer Calbindin+ mDA VTA neurons

Birth-dating experiments have demonstrated that mDA neurons are born sequentially with more lateral mDA neurons that project to the striatum born first and the medial mDA neurons projecting to the PFC, born later in development (25,26). To examine how the altered differentiation dynamics in the mutant embryos affects the distribution and subtype composition of mDA neurons in the postnatal brain we examined the expression of Girk2 and Calbindin (Calb1), markers expressed predominantly in the lateral SNc mDA neurons and the medial VTA mDA neurons respectively at postnatal (P) day 15. Both subpopulations of TH+ mDA neurons can be identified in the VM of both WT and mutant brains, with the Calbindin+/TH+ cells clustered around the ventral midline whereas the Girk2+/TH+ cells are distributed more laterally (fig 5). A small sub-section of mDA neurons expressing both Girk2 and Calbindin were found largely restricted to the dorsolateral VTA in the transition zone between the SNc and VTA. The lama2$^{-/-}$ brains were found to be smaller at P15 (fig S11) and to have fewer TH+ mDA neurons in the midbrain compared to WT littermate controls (fig 5A). The loss of neurons was particularly apparent in the mediocaudal VM (fig S12), suggesting that the later-born medial mDA neurons were preferentially depleted in the mutant mice. Quantifying Girk2 and Calbindin expression confirmed this, revealing a reduction in the number of TH+ Calbindin+ (later-born) mDA neurons with no significant decrease in the number of Girk2+ TH+ (early-born) mDA neurons (fig 5B-C). As expected, quantification of the ratio of TH+ mDA neurons that are either Girk2 or Calbindin+ in the mutant brains revealed proportionately more Girk2+ and fewer Calbindin+ mDA neurons (fig 5D). These results suggest that premature depletion of the mDA progenitor pool, due to a smaller progenitor domain combined with accelerated differentiation and increased apoptosis, leads to a loss of late-born Calbindin positive mDA neurons creating an

imbalance between these two populations of dopaminergic neurons in the VM of *lama2*$^{-/-}$ brains.

Discussion

In this study, we describe the expression and distribution of laminin isoforms in the mouse and human VM and then identify a dual functional role for one of these, lm-α2, in controlling cell cycle exit and survival in the mDA neuron lineage. We propose a model whereby the lm-α2 rich environment, found early in development, sustains VM progenitor proliferation via an integrin-dependent pathway. As lm-α2 expression diminishes, cells are able to exit from the cell cycle and differentiate with low levels of lm-α2 then promoting the survival of progenitors through dystroglycan receptors. In mutant lama2$^{-/-}$ mice, the loss of lm-α2 protein results in a smaller mDA progenitor pool that is subsequently depleted by a combination of increased cell death and premature differentiation, leading to a defect in late generated mDA neurons of the VTA subtype.

Previous studies examining lm expression in the brain describe the murine neocortex as being rich in lm-α2 and lm-α4, with expression greatest at the ventricular surface (3,27). Moreover, the subventricular zone, one of two adult stem cell niches in the CNS, was found to be a lm-rich environment compared to neighbouring non-neurogenic regions suggesting lms may play a role in regulating NSC self-renewal (28). Consistent with this, we find the presence of high levels of interstitial lm-α2 in the early human embryo during a period of rapid growth, after which levels decline. Three other laminin isoforms, lm-α1, lm-α4 and lm-α5 were largely restricted to the basal lamina. Together, our immunostaining and analysis of a previously-published scRNA-seq dataset reveals lm expression to be highly specific and dynamically regulated over the course of development.

Several independent studies have implicated the lm-integrin interaction in controlling NSC proliferation in both the embryonic cortex and in the adult stem cell niches (29–32). Additionally, in pathological conditions characterised by rapid proliferation such as glioblastoma multiforme (GBM), the cellular niche has been described to be rich in lm-α2 whilst the glioblastoma stem-like cells that drive tumour growth are enriched with integrin α6 and α7 (33–35). Disruption of the lm-integrin interactions in GBM significantly impaired tumour growth (33–35). Given our results showing the expression of lm-α2 in mDA progenitors maintains cells in a proliferative state, it would be interesting to test the hypothesis that a common mechanism underpins the lm-integrin driven NSC proliferation in embryonic, adult and pathological conditions. Identifying downstream mechanisms as well as positive and negative regulators of lm expression could be important not only to control proliferation in cancer, but also in a regenerative context, offering the potential to counteract the age-dependent decline in NSC self-renewal.

Over the course of development, laminin expression diminishes and is restricted to the ventricular zone, limiting the cells it can interact with to those rapidly dividing progenitors juxtaposed to the surface of the ventricle. The interaction with lm-α2 in this region prevents exit from the cell cycle and differentiation. As predicted from this hypothesis, in the complete absence of lm-α2 precocious mDA neurons can be seen at both the basal surface and ectopically at the ventricular surface. An increase in the number of apoptotic cells in the interface between the ventricular and intermediate zone can also been seen, reflecting the requirement for lower concentrations of lm-α2 to maintain the survival of the newly-

postmitotic cells in this region. A previous study identified that lm-α5 driven YAP activation promotes the survival of mDA neurons in the marginal zone (36), at a later differentiation stage compared to the one described in this study. Interestingly, lm-α2 has also been reported to activate YAP and further upregulate the expression of lm receptors such as integrins and dystroglycan in Schwann cells (37), suggesting a possible interaction between these two lm-activated pathways and a general role of lms in controlling cell survival at different developmental stages along the same lineage. Indeed, it is perfectly feasible that the lm511 present in the VM may be compensating for the loss of lm211, masking the extent of the phenotype.

We have previously shown that the principal lm receptor, integrin β1, is capable of regulating both NSC self-renewal and differentiation in the chick midbrain via distinct mechanisms (38). Meanwhile, the relative abundance of the lm protein has been shown to control the balance between quiescence and activation in epidermal stem cells (39). Our present results extend these prior studies by showing a concentration-dependent effect of lm-α2 controls the proliferation and survival of human progenitors, at least in part, by sequential receptor engagement with integrins and dystroglycan. It is interesting to note that a similar interplay between the two receptors has been identified in lm-mediated pancreatic β-cell development (40). Moreover, αβ integrins and dystroglycan have been shown to act sequentially during Schwann cell development in the peripheral nervous system (PNS), activating different signalling pathways during axonal sorting (41). In agreement with these previously published studies, the data presented here suggests that the two lm receptors are responsible for distinct steps during mDA development. How lm-211 is able to regulate these specific processes in a concentration-dependent manner remains an open question. One possible explanation may lie in receptor affinities. Previously published solid-phase binding assays of lm211 with dystroglycan and a number of α-integrins demonstrated that the dystroglycan interaction is an order of magnitude greater than with integrins (42,43), and so is likely to be favoured at the lower lm concentrations later in the developmental process.

Mutations affecting lm-α2, dystroglycan and more rarely integrin α7 result in CMD, a clinically and genetically heterogeneous group of disorders affecting primarily muscle and brain. Neurological deficits that includes structural malformations and cognitive impairment are most abundant in patients carrying mutations affecting dystroglycan (44,45). However, a number of patients carrying a lama2 mutation also report neurological abnormalities including brainstem hypoplasia (8,9). In the lm-α2 null embryos described in this study, we found a VM significantly reduced in size likely due to the impairment of proliferation, premature differentiation and increased cell death resulting in a depletion of the progenitor pool; a phenotype reminiscent of integrin β1 mutant mice in which cerebellar granule cell precursors cease proliferating and differentiate prematurely (46). Interestingly, impaired Shh signalling contributed to the premature differentiation in integrin β1 mutant mice following disruption of the integrin-laminin-Shh complex (46). Moreover, Shh-proteoglycan interactions were previously shown to be necessary for regulating proliferation but dispensable for tissue patterning (47). As we find a defect in proliferation but with correct patterning and a subtle delay in *Shh* expression in lm-α2 null embryos, a contribution of Shh signalling to the proliferation phenotype cannot be excluded.

A well-established consequence of premature differentiation and depletion of the progenitor pool is a loss of later-born neurons (48–50). In accordance with these studies, we found a disruption of mDA neuron subtype composition, with a reduction in the later-born, Calbindin positive mDA neurons of the VTA. The mDA neurons of the VTA project to the caudal brainstem, hippocampus and PFC and play an important role in a number of processes including cognition, motivation and attention related behaviour. Indeed a subset of these late-born mDA neurons project almost exclusively to the GABAergic interneurons of the PFC and regulate perseveration-like behaviour (51). This loss of late-born neurons may contribute to the cognitive defects described in the few published reports of behavioural and psychiatric problems in MDC1A patients. Of potentially more importance, however, is the possibility that abnormalities in the laminin-dystroglycan-dystrophin pathway caused by dystrophin mutations in Duchenne muscular dystrophy (DMD) patients may, by altering mDA neuron production, contribute to the high prevalence for ADHD and autism spectrum disorder (ASD) described in a recent study on 130 Duchenne muscular dystrophy (DMD) patients (10). In keeping with this, a separate study reported a reduction in $GABA_A$ receptor clustering in the PFC – a target of the late-born mDA neurons – of 14 DMD patients (52). Given the roles we have ascribed to lm-α2, a more thorough behavioural and psychiatric analysis of MDC1A patients would be of great value and provide some support to the notion that a dopamine imbalance underpins the behavioural deficits observed in muscular dystrophies.

Methods

Human and mouse tissue processing

OCT Tissue-Tek embedded human foetal midbrain tissue (5-10 pcw) was obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR). Sequential coronal sections covering the length of the mesencephalon were collected on superfrost glass slides (ThermoFisher) using a cryostat (ThermoFisher).

All mouse experiments were conducted following the procedures approved by Roswell Park Institute Animal Care and Use Committee (UB1188M, UB1194M, and UB1196R). The protocols follow the guidelines of the “Guide For The Use of Laboratory Animals,” National Research Council, National Academy Press, Washington D.C., 1996. All animals used in this work were congenic into the C57/BL6N background and genotyping of *Lama2* mutant mice was done by PCR of tail genomic DNA. Briefly, PCRs were done 45s at 95°C, 45s at 50°C, and 60s at 72°C for 30 cycles. The primers used were as follows: 5’-CCCGTGATATTGCTGAAG-3’, 5’-CCTCTCCATTTTCTAAAG-3’ and 5’-CAGGTGTTCCAGATTGCC-3’. The lama2 mutant used in this study was the dy3k mutant comprising a complete knockout of the laminin alpha 2 protein (53).

To obtain embryos, matings were set late in the evening and plugs checked the next morning before 9 AM with those that were positive designated as E0.5. At E10.5, E12.5 and E14.5, pregnant mice were sacrificed. E10.5 whole mount embryos and the dissected heads of E12.5 and E14.5 embryos were fixed in 4% PFA overnight at 4°C.

From the EdU labelling studies, pregnant mice were subcutaneously injected with 50mg/kg of 5-Ethynyl-2′-deoxyuridine (EdU, Sigma 900584) at E11.5. Embryos were dissected 24 hours later at E12.5.

Embryos were washed in PBS (3×5mins) and incubated in 15% sucrose (Sigma) in PBS at 4°C overnight. The embryos were then placed in embedding solution that consisted of 15% sucrose and 7% gelatin (Sigma) in PBS at 37°C for a period of 2 hours. Finally, the embryos were placed in moulds and orientated for coronal sections and snap frozen in liquid N_2 and stored at -80°C. 10µm serial sections spanning the length of the midbrain were taken from each sample. Two embryos from 2-3 different litters were analysed for each time-point (N=4-6). Individual figure captions make clear the N used for each experiment.

In the case of P15 brains, animals were anesthetized with 20 mg/ml Avertine (2,2,2-tribromoethanol, Sigma, T48402) and sequentially perfused through the left ventricle with ice cold 1X PBS and 4% PFA. Next, the brains were dissected and post fixed in 4% PFA overnight at 4˚C and transferred to 30% sucrose for 48 hours at 4°C and snap frozen in liquid N_2. 20µm sections were collected on glass slides. Two brains from two different litters were examined for both mutant and control animals (N=4).

Cell cultures

Undifferentiated RC17 ES cells (passages 23-58, Roslin Cells, hPSC reg #RCe021-A) were maintained in E8 media (A1517001) on Geltrex (1%, 12760021) coated plates and passaged weekly with EDTA (0.5 mM). To start differentiation (day 0), hESC colonies were detached using EDTA (0.5 mM) and placed in non-treated 60mm culture dishes in differentiation media consisting of DMEM:F12/Neurobasal (1:1), N2 supplement (1:100), B27 supplement (1:50), SB431542 (10uM, Tocris Biosciences), rhnoggin (100 ng/ml, R&D), SHH-C24II (200 ng/ml, R&D) and CHIR99021 (0.9 µM, Tocris Biochem). Media was changed once on day 2. The resultant embryoid bodies were collected on day 4 and placed on polyornithine (PO), fibronectin (Fn) and laminin (lm) coated plates in reduced N2 (1:200) and B27 (1:100) condition. Growth and patterning factors were removed on day 9 with the cultures kept in DMEM:F12/Neurobasal (1:1), N2 supplement (1:200), B27 supplement (1:100). On day 11, the cell clusters were dissociated to single cells with accutase and replated onto dry PO/Fn coated plates for differentiation in Neurobasal, B27 (1:50), BDNF (20 ng/ml), GDNF (10 ng/ml), AA (200 µM), db-cAMP (0.5 mM, Sigma). All culture reagents are from Invitrogen unless otherwise stated here or previously.

Experiments were conducted from day 14, where cultures were treated with soluble lm isoform (BioLamina) or vehicle, with each media change, for stated period of time. Media was changed every third day until fixation in 3.7% PFA for 30 minutes. Where stated, an EdU pulse was administered for 24 hours prior to fixation to identify dividing cells.

Receptor blocking antibodies or isotype controls were added to the culture media at 5 µg/ml (10 µg/ml for dystroglycan) and refreshed every 3 days with each media change. Antibodies used were: integrin α3 IA3 (R&D MAB1345-SP), integrin α6 GoH3 (R&D MAB13501), integrin α7 6A11 (LifeSpan Biosciences, LS-C179572), Dystroglycan IIH6C4 (Millipore, 05-593), mouse-IgM (ThermoFisher, 02-6800), rat-IgG2a (ThermoFisher, 02-9688), mouse-IgG1 (ThermoFisher, 02-6100).

Immunofluorescence, microscopy & image quantification

List of antibodies used, suppliers and dilutions are provided in table S1.

Human and mouse sections were boiled in antigen unmasking solution (Vector Labs) and pre-incubated at room temperature for 1 hour in blocking solution containing 10% normal donkey serum (Millipore), 1% bovine serum albumin (Sigma), 0.2% Triton X-100 (Sigma). Sections were incubated with primary antibodies diluted in blocking solution at 4°C overnight followed by washing with blocking solution (3×15mins). Sections were then incubated with fluorophore conjugated secondary antibodies (Invitrogen) diluted 1:1000 in blocking solution for 1 hour at room temperature, followed by washing (3×15mins), hoechst nuclear counter stain (10mins at room temperature) and mounted (Fluoromount, SouthernBiotech).

The Operetta high-content microscope (PerkinElmer) was used for automated image-acquisition of cell cultures that were then quantified using automated quantification pipelines developed using Columbus Image Analysis Software (PerkinElmer).

Meanwhile, tissue sections (both embryonic and P15) were imaged on an SP8 confocal microscope (Leica) and quantified manually using Fiji CellCounter plug-in. For quantification of embryonic tissue, adjacent sections were used for labelling (FoxA2, Ki67, Nurr1 and TH) and 5 sections spanning the anterior-posterior axis were counted per stain and sample. For P15 brain, TH+ mDA neurons were quantified in sections at three rostrocaudal levels (Bregma: -2.92mm, -3.40mm and -3.88mm). TH+, TH+ Girk2+ and TH+ Calbindin+ cells were counted bilaterally for each level. Tissue sections were quantified whilst blinded to genotype.

RNAscope

RNAscope mRNA detection was performed according to the manual, using the RNAScope 2.5 HD reagent Kit-RED (ACD, 322350). PFA fixed sections (10µm) were dehydrated in an ethanol gradient (50%, 70% and 100%) and incubated with H_2O_2 for 10 min. Sections were washed in deionized water and then boiled for 5 minutes in antigen retrieval solution then transferred to a dish containing distilled water. After washing, slides were rinsed in fresh 100% ethanol and air dried. RNAscope Protease plus was applied to the slides and incubated for 30 mins at 40°C. Slides were then washed in distilled water with slight agitation. The appropriate RNAscope probe (Shh: cat no 314361, Wnt1: cat no 401091) was then applied to the slide and incubated for 2 hours at 40°C. Sections were then washed in wash buffer and the colour reaction was performed according to the user manual. Sections were counterstained for 1 min with haematoxylin (Scientific Laboratories, GHS132-1L), and the blue reaction was performed using 0.02% ammonia water. Sections were dried at 60 °C and mounted.

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Acknowledgements

The authors would like to acknowledge Drs Eoghan O'Duibhir and Bertrand Vernay for assistance with imaging and image quantification software. The human embryonic and fetal material was provided by the Joint MRC/Wellcome Trust (MR/R006237/1) Human Developmental Biology Resource (www.hdbr.org). CffC supported by EU FP7 Neurostemcellrepair grant no 602278 and the Wellcome Trust Senior Investigator Award. EA supported by Swedish Research Council (VR 2016-01526), Swedish Foundation for Strategic Research (SB16-0065), European Commission (NeuroStemCellRepair), Karolinska Institutet, Hjärnfonden (FO2017:0059), Cancerfonden (CAN 2016/572) and SFO Strat Regen (SG-2018). The authors declare no competing interests, financial or otherwise.

Author contributions

Conceived and designed experiments: MA, EA and CffC. Performed experiments: MA. Analysed data: MA. Contributed reagents/materials: LM and MLF. Wrote the paper: MA, EA and CffC.

Fig 1: Lm-α2 expression in the human ventral midbrain at 6 and 10 pcw.
Lm-α2 expression (antibody: C13065) is enriched in the human VM at 6pcw, when the hVM is composed of Ki67+ FoxA2+ progenitors. Expression diminishes by 10pcw when the hVM contains few Ki67+ cells and post-mitotic Nurr1+ mDA neurons are established. scRNA-seq of the hVM suggests pericytes (hPeric) are the principle source of Lm-a2 (Right axis shows absolute molecule counts). Scale bar 50 μm.

Fig 2: Lm211 regulates proliferation and differentiation in a concentration dependent manner.
(A) Immunostaining of day 28 hES derived mDA cultures treated with lm211 over a concentration range of 0.5-12 μgml^{-1}. Quantification of EdU (B) and TH (C) staining showing a significant increase in proliferating (EdU+) cells at lm211 concentrations of over $4\mu gml^{-1}$ which is associated with a reduction in TH+ mDA neurons (One-way ANOVA with Tukeys post-test, $p<0.0001$, n=3). (D-G) Time series examining proliferation (D) differentiation (E, F) and survival (G) at days 21, 28 and 35 of culture at two different lm211 concentrations. mDA progenitors remain EdU+ at day 28 and 35 in the lm211-rich ($4\mu gml^{-1}$) environment, resulting in a significant reduction in the number of post-mitotic mDA neuroblasts (Nurr1) and neurons (TH). No significant differences in aC3 staining between the two lm211 concentrations (unpaired, two-tailed t-test, * $p<0.0001$, n=3). (H) Representative images of day 35 cultures following lm211 treatment. Scale bar 50 μm in all images.

Fig 3: Concentration-dependent effects of lm211 are mediated by distinct receptor engagement
(A) Immunostaining of day 28 cultures treated with lm211 ($4\ \mu gml^{-1}$) in combination with integrin blocking antibodies and a control condition of lm211 ($1\ \mu gml^{-1}$). Quantification of proliferation (B) and differentiation (C) following blocking of integrin a-subunit demonstrating that blocking both a6 and a7 integrins reverses the effect of the lm211-rich environment. (D) Images of day 28 cultures treated with lm isoforms ($1\ \mu gml^{-1}$) and dystrolgycan blocking antibody or isotype control. Quantification of proliferation (E) and differentiation (F) demonstrating blockade of dystroglycan reverses the gain in mDA neurons seen on lm211 compared to lm111 without effecting proliferation. *$p<0.0001$, unpaired two-tailed t-test, n=3. Scale bar 50 μm in all images.

Fig 4: Lm-α2 null VM is reduced in size with fewer cells and exhibits premature differentiation and depletion of progenitor pool
Representative images and quantification of wild-type and mutant VMs at (A-D) E10.5 and (E-M) E12.5. At E10.5, mutant embryos display a smaller ventral domain indicated by FoxA2 staining. Fewer FoxA2+ cells are Ki67+ in the $lama2^{-/-}$ embryo (B) with increased numbers of post-mitotic mDA neuroblasts (Nurr1+) and mDA neurons (TH+) quantified in (C) and (D) respectively. By E12.5, there remains fewer proliferating FoxA2+ cells (F) and Lmx1a+ cells (G) in the VM of mutant embryos. The intermediate and marginal zones of the mutant VM contain fewer Nurr1+ (H) and TH+ (I) cells whilst there is an increase in apoptotic (aC3+) cells at the ventricular zone and intermediate zone border in the absence of lm-α2. Whilst there is a reduction in the number of Lmx1a+ mDA progenitors (L), the number of Lmx1a+ Ngn2+ cells remains the same resulting in a greater proportion of progenitors undergoing neurogenesis (12.51 ± 0.89% (WT) vs 20.73 ± 3.49 % ($lama2^{-/-}$)) in the mutant embryos. EdU labelling confirmed this with a significant increase in the number of EdU+ Nurr1+ cells in the mutant embryos (512 ± 41 (WT) vs 663 ± 88 % ($lama2^{-/-}$)) whereas there was a significant decrease in the number of Lmx1a+ EdU+ cells that are Nurr1- (201 ± 35 (WT) vs 116 ± 26 ($lama2^{-/-}$)). N=4-6, two tailed unpaired t-test, *$p<0.01$, scale bar 50μm.

Fig 5: The midbrain of lm-α2 null brains contains fewer late-born Calbindin+ mDA neurons of the VTA
Postnatal brains (P15) of wild-type and mutant mice showing fewer TH+ mDA neurons (A) in the $lama2^{-/-}$ brains. In quantifying mDA subtype, there is a modest reduction in the number of Girk2+ mDA neurons (B) but a dramatic loss in Calbindin+ mDA neurons (C) located medially in the VM. In normalising for the number of mDA neurons (D), there is a significant increase ($p=0.0033$) in the proportion of Girk2+ TH+ mDA neurons in the mutant mice concomitant with a reduction ($p=0.0105$) in Calbindin+ TH+ mDA neurons. N=3 (WT), 4(KO), unpaired two-tailed t-test. Scale bar 100μm unless stated.

Fig S1: Lm-α chain immunohistochemistry in human VM
(A) Lm-α1 expression is restricted to the basement membrane surrounding the basal surface neural tube. Lm-a3 is not expressed in the human VM. Lm-a4 is restricted to the basal laminae of blood

vessels at both 6 and 10 pcw. Meanwhile lm-a5 is expressed on both the ventricular and basal surfaces of the VM at 6 pcw as well as some interstitial expression. (B) scRNA-seq data of individual lm-a chains showing cell types for gene expression in human development. Right axis shows absolute molecule counts. (C) Lm-α2 expression in the mouse VM at E10.5-E14.5 displays a similar expression pattern as that seen in the human embryo. Expression can be seen to diminish over time with negligible positive expression at E14.5.

Fig S2: Differentiation protocol and patterning of hES cells into mDA progenitors
(A) schematic of hES differentiation protocol with lm treatment at day 14 till fixation. (B) Immunostainings of day 11 cultures showing cultures to be triple positive for the mDA progenitor markers FoxA2, Lmx1a and Otx2. Cultures are negative for the pluripotency marker Oct4, forebrain marker Pax6 and the lateral domain marker Nkx6.1. (C) TH+ Neurons at day 35 showing positive immunoreactivity for a panel of markers (Nurr1, Pbx1a, DDC, Calb1, Girk2) illustrative of bona fide mDA neurons. Scale bar 50μm.

Fig S3: Lm isoform specificity in regulating mDA progenitor proliferation and survival
(A) Representative images of mDA cultures at day 28 exposed to lm211 at 1 and 4 μgml^{-1}, staining for proliferation (EdU) and neurons (TH). At low concentrations (1 μgml^{-1}), no significant difference in proliferation (B) is detected between any of the lm isoforms whilst an increase in TH+ mDA neurons (C) is observed on lm211. At high concentrations (4 μgml^{-1}), no differences are detected in the number of mDA progenitors that are EdU+ (D) or TH+ (E). *p<0.001, ANOVA Tukeys post test, N=3

Fig S4: Expression of lm receptors
Integrins α6, α7, β1 and Dystroglycan are expressed in the mouse VM and on hES derived mDA progenitors at day 14 of culture. scRNA-seq of the hVM identifies radial glial (hRgl1-3) and VM progenitors (hProg) positive for lm receptor expression. Right axis shows absolute molecule counts. Scale bar 50μm.

Fig S5: Specificity of laminin-receptor interactions
(A) Integrin α6 and α7 blocked with antibodies in the presence of 4 μgml^{-1} of lm111, lm411 and lm511. When cultures are exposed to the integrin blocking antibodies, the lm-driven increase in proliferation is abrogated suggesting that the integrin receptors are mediating the proliferative effects of lm. (B) Blocking the α-dystroglycan receptor with no exogenous lm211 does not effect the number of TH+ neurons generated. N=3, two-tailed unpaired t-test, *p<0.001.

Fig S6: Lm-α2 expression in the wild-type and lama2$^{-/-}$ mouse VM
No Lm-α2 expression can be detected in the lama2$^{-/-}$ embryos confirming the knock-out.

Fig S7: Lama2$^{-/-}$ exhibit defects in growth but normal patterning
(A) Cross-sections of wild-type and lama2$^{-/-}$ mesencephalon at E10.5 (scale bar 100 μm) with the mutant mesencephalon significantly smaller in area (B). The dopaminergic domain consisting of FoxA2, Lmx1a and Corin (scale bar 50 μm). Fewer FoxA2 cells are present in the lama2$^{-/-}$ midbrain (D). The area of each domain was calculated and normalised to the area of the ventricle. No significant differences were detected in the size of the normalised FoxA2 (E), Lmx1a (F) and Corin (G). Nkx6-1+ cells can be seen laterally and are induced in both the mutant and wild-type embryo at E10.5 (scale bar 50 μm) (H). Potential ectopic expression and a delay in the lateral expansion and medial inhibition of Shh expression in the mutant embryos at E12.5 can be seen. Wnt1 expression is comparable to wild-type and previously published reports (scale bar 100 μm) (I). Dopaminergic domain remains smaller at E12.5 (scale bar 50 μm) (J) in the mutant embryo compared to wild-type, consisting of fewer FoxA2 cells (K) but when normalised for ventricle size, there are no significant differences in FoxA2 (L), Lmx1a (M) or Corin (N) domain size. N=4-6, two-tailed unpaired t-test.

Fig S8: Ectopic mDA neurons in the VM of lama2$^{-/-}$ embryos
TH+ mDA neurons can be seen lining the ventricular surface of lm-α2 null embryos at E10.5 (orange arrows). Ectopic mDA neurons (orange arrowhead) at the ventricular surface (dashed line) continue to be observed at E12.5. Scale bar 50 μm.

Fig S9: Reduced mDA neurons in the VM at E14.5

Significantly fewer TH+ mDA neurons in the VM of mutant embryos compared to wild-type littermate controls. N=6, two-tailed unpaired t-test, scale bar 50 µm.

Fig S10: Development of non-mDA VM neurons compromised in lama2$^{-/-}$ mutants.
Brn3a+ red nucleus neurons, derived from FoxA2+ progenitors, are significantly reduced in the mutant embryos compared to wild-type littermate controls at E14.5. N=6, two-tailed unpaired t-test, scale bar 50µm.

Fig S11: Smaller mutant brains compared to wild-types at P15
Lama2$^{-/-}$ brains are significantly smaller than WT littermate controls, quantified via mass. N=4, two-tailed unpaired t-test.

Fig S12: VM of wild-type and mutant P15 brains showing reduced number of neurons
Large panels are images of the whole VM from wild-type and lama2$^{-/-}$ P15 brains along the rostral-caudal axis showing reduced TH+ immunoreactivity (white) in the mutant brains (scale bar 100 µm). Images to the side are expanded view of the red boxes displaying TH (white) and Calb1 (red) (scale bar 50 µm). Fewer TH+ Calb1+ double positive cells can be seen in the mutant brains, particularly in the more caudal sections.